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INHIBITION OF THE HIV REV TRANSACTIVATOR: A NEW TARGET FOR THERAPEUTIC INTERVENTION

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4. Rev as target for therapeutic intervention

As described above, Rev function is essential for viral replication. No cellular homologs of Rev have been described so far. Several steps are required for Rev function: binding to the RRE, oligomerization of Rev monomers, and interaction with cellular factors from the nuclear transport machinery. Each of these steps provide potentially specific targets for therapeutic intervention, and the fact that the structural contacts for RRE binding, as well as the role of Rev in nuclear export and its interaction with cellular proteins are now well understood makes this protein a very attractive therapeutic opportunity for the treatment of HIV infection and AIDS. Even though Rev represents an excellent viral target, no anti-Rev compounds have yet entered clinical trials, although some clinical studies have been initiated using gene therapy approaches involving Rev (see 68 for review of these efforts). In this Section, I will review the results of gene therapy, antisense and drug discovery efforts focused on the Rev protein.

4.1 Rev as a target for gene therapy

The resistance of HIV to anti-viral drugs, especially in the early days of single-drug regimes, has prompted a search for alternative methods of therapy. One approach has been gene therapy, meaning the transfer of antiviral genes to infected cells. This strategy is based on the notion that these "therapeutic" genes will render target cells resistant to HIV replication. Gene therapy can be based on the expression of suppressor proteins, or on expression of anti-viral RNA or DNA molecules. Some excellent review articles on gene therapy of AIDS have been published in the past few years (68-71). An extensive review of the anti-HIV gene therapy approaches is beyond the scope of this article,

and I will focus on strategies involving Rev.

4.1.1 Protein-based suppressors of Rev function

One of the most advanced protein-based approaches involves the Rev mutant M10, a trans-dominant negative mutant with amino acid substitutions at positions 78 and 79 in the NES/activation domain, that retains the ability to bind to the RRE and multimerize, but is unable to effects its role in transport of pre-mRNAs (25, 72). Because of its trans-dominant negative phenotype, the M10 protein inhibits HIV replication when expressed in stable cell lines (73-76). In HIV infected patients Rev M10-transduced T cells showed increased survival compared to T cells transduced with a vector expressing a deletion mutant of Rev M10 (77). High levels of M10 are required to inhibit viral replication in primary cells, and the choice of vectors is therefore critical to the success of gene therapy (78). A Phase I clinical trial taking into account these parameters has been initiated by Systemics, Inc. (Palo Alto, CA).

Another protein-based strategy that has been explored is expression of an anti-Rev single-chain antibody (79). This single-chain antibody, or SFv, was expressed from a construct consisting of both light and heavy chain variable regions of an anti-Rev monoclonal antibody. Intracellular expression of this SFv resulted in a level of inhibition of HIV replication comparable to that shown with the Rev M10 transdominant mutant (79). This antibody appears to sequester Rev in the cytoplasm, thus preventing it from exerting its function in nuclear transport.

4.1.2 Intracellular expression of RNA-based Rev inhibitors

A large portion of the anti-HIV gene therapy efforts is based on RNA-based suppressors of viral replication, like ribozymes and RNA decoys. Ribozymes are RNA molecules that can be engineered to cleave RNA at specific sites (80). Retroviral vectors expressing hammerhead ribozymes targeted against different regions of the HIV genome have been shown to inhibit viral replication in transduced cells (81-83). A hammerhead ribozyme targeting the common exon of the Tat and Rev genes has been shown to inhibit HIV replication in a human T cell line (84). Because ribozymes are extremely sequence specific, mutations in the virus would rapidly result in resistance. To address this concern, combination strategies with ribozymes that target different sites, or with ribozymes together with other antiviral genes, such as RNA decoys, have been proposed. In fact, a fusion molecule consisting of a ribozyme targeting the U5 region of the HIV LTR and an RNA decoy representing stem-loop IIB of the RRE, has been shown to be more efficient than ribozymes or RNA decoys alone (85, 86). The expression of antisense RRE decoys in retroviral vectors is also being explored as of potential therapeutic value (87-89). The effects of stable expression of antisense RNA targeting the Rev, Tat, and Vpu genes on viral replication has also been investigated, and showed to be of limited efficacy (90, 91).

Although gene transfer for the treatment of HIV infections is an attractive alternative or complement to the use of antiviral drugs, it is still not a reality, and many problems related to gene delivery and level of expression remain to be solved (69). More classical antiviral approaches, such as drug discovery, are being pursued, extending the efforts towards other viral targets, and one of these is Rev. The next Sections will describe antisense and drug screening targeting the Rev protein, that do not involve gene transfer.

4.2 Inhibiting Rev function via antisense oligonucleotides and other nucleic-acid molecules

The antisense RNA strategy was inspired by a naturally occurring mechanism of gene regulation in prokaryotes (92). The specificity of Watson-Crick base pairing made antisense molecules very attractive as potential therapeutic agents. A vast amount of literature exists on the application of this strategy to human diseases, including viral infections (reviewed in 93). Both unmodified and modified antisense oligonucleotides directed against various HIV RNA sequences have been shown to inhibit viral replication, both in a sequence-specific and in a non-sequence specific manner. A synthetic phosphorothioate oligodeoxynucleotide targeting Rev mRNA has been shown to have antiviral activity in chronically infected cells, inhibiting HIV replication by 80% at 25 μM (94), possibly through inhibition of translation. Since very early on, oligonucleotides complementary to the RRE sequences were shown to have the capability of disrupting Rev-RRE binding in vitro (95), several modified oligonucleotides targeting different stem-loops of the RRE have been tested for inhibition of viral replication (96, 97), and found to inhibit viral replication in a specific manner.

A novel nucleic acid-based approach towards inhibition of HIV infection by blocking Rev function has been the use of decoy RNA-DNA chimeric oligonucleotides containing the high affinity 13 nucleotide "bubble" structure of stem-loop IIB (see Figure 3) (98). These chimeric decoy bound the RRE with high affinity in vitro and were shown to inhibit HIV replication 40-70% at ~10 mM, using various assays (98).

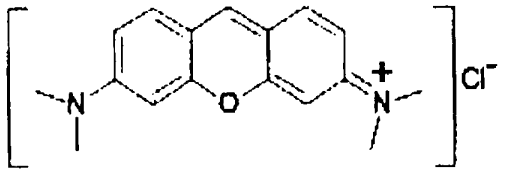
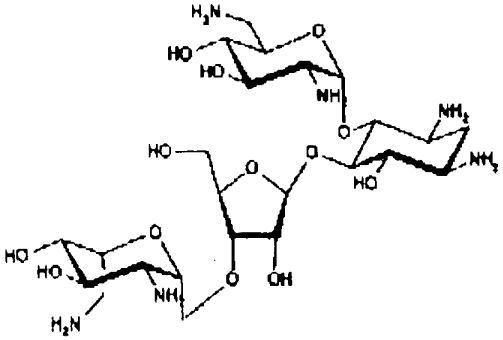
In spite of the enthusiasm generated by the use of phosphorothioate oligonucleotides in the area of viral diseases, to date these strategies have met with limited success and significant issues remain in their potential use as therapeutic agents, including efficacy, cell permeability, delivery and cost. Because of the present limitations of both gene therapy and nucleic-acid-based antivirals, it is important that traditional approaches, such as screening for compounds with anti-Rev activity, are explored. The next Section will review the low molecular weight compounds and natural products that inhibit Rev.

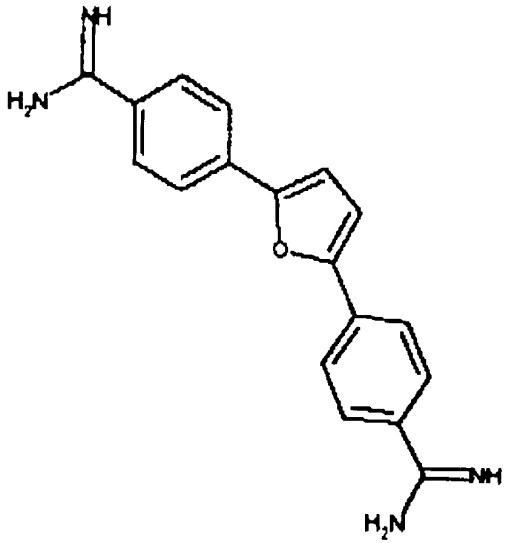
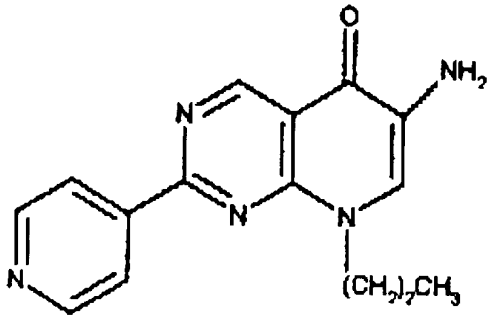
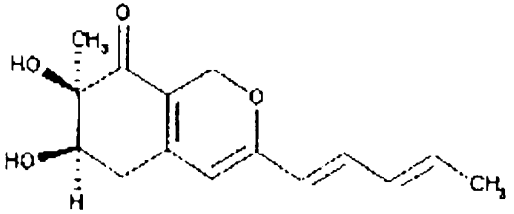
4.3 Low-molecular weight compounds and natural products that inhibit Rev

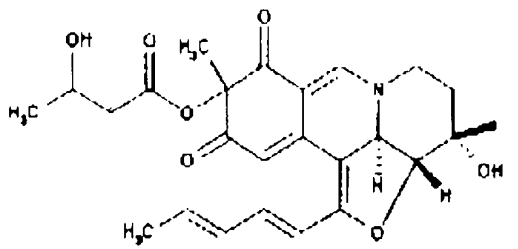
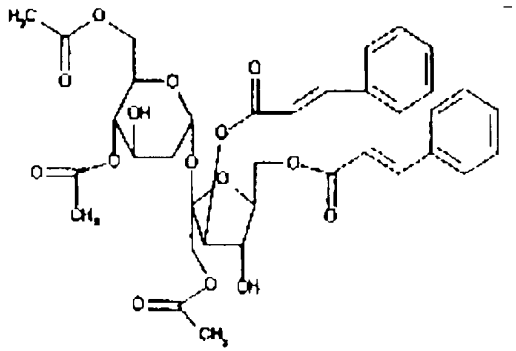
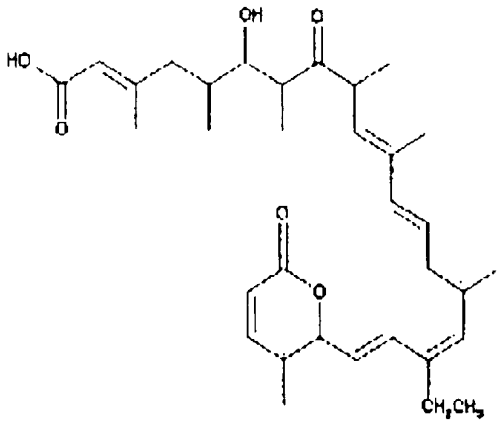
Rev has been considered a promising target for therapeutic intervention of HIV infections since it was proven to be

essential for HIV replication. The earliest attempts at intefering with its function were based on antisense technology, in the late 1980s (see previous Section). In the past few years, knowledge of the mechanism of action of Rev has increased rapidly, and it became clear that Rev offers several molecular targets for drug discovery. The very specific Rev-RRE interaction have been a preferred target for drug discovery, since it has no cellular counterpart. At the same time, other groups have focused on cell-based assays that would allow discovery of a drug that acts at the level of the interaction of Rev with the cellular transport machinery, as well as at the RNA-binding level. The next sections will describe the compounds and natural products that have been found to interfere with Rev function, as well as their potential usefulness as therapeutic agents. A list of these agents is presented in Table 1.

Table 1. Low molecular weight inhibitors of Rev function. The structure of the compounds described so far as Rev inhibitors is shown, as well as the molecular target and their effect on HIV replication assays.

Compound		**Molecular target**	**Inhibition of HIV replication**
Pyronin Y		RRE binding (Intercalating agent)	No
Neomycin B		RRE binding	85% at 2.5 mM
Diphenylfuran		RRE binding	Not tested

Derivatives		(Intercalating agent)	
WIN-49611		Unknown, NOT RRE binding	Yes, IC_{50}=3.5 μM
Harziphilone		RRE binding	NO
Fleephilone		RRE binding	NO

			
Niruriside		RRE binding	NO
Leptomycin B		Inhibition of nuclear export of Rev	Yes, IC_{50}=2-6 nM

4.3.1. Intercalating agents and other RNA-binding compounds

The first approaches towards anti-Rev drug discovery focused on the Rev-RRE interaction. Because Rev binds to

an RNA target, intercalating agents with specificity or preference towards RNA were investigated as potential Rev-RRE inhibitors. The intercalating dye pyronin Y was reported to completely block the formation of the Rev-RRE complex *in vitro*, at low μM concentrations (99). In agreement with previous reports that Rev-RRE binding is a pre-requisite for oligomerization (13, 19, 20) this intercalating agent also block the formation of multimeric complexes. Despite these strong *in vitro* effects, the dye failed to inhibit HIV replication in cytoprotection assays, in part because of its high levels of cellular toxicity (99). This result was not altogether surprising since pyronin Y is known to intercalate DNA in addition to RNA. Other intercalating agents, derivatives of diphenylfuran, were also reported to inhibit Rev-RRE interaction, by causing a conformational change in the RRE (100). Although these agents can be useful as probes to investigate the precise mechanism of Rev-RRE binding, intercalating agents are clearly not attractive molecules from a therapeutic point of view, because of their many toxic and mutagenic effects.

Non-intercalating compounds with previously known RNA binding properties were also candidates for inhibition of Rev-RRE binding. In this group of molecules, the aminoglycoside antibiotic neomycinB and some of its analogs were reported to disrupt Rev binding to the RRE in a specific manner (40, 101). Aminoglycoside antibiotics are known to act at the level of prokaryotic ribosomes, disrupting mRNA translation by binding to 16S RNA (102,103). In addition to binding to bacterial 16S RNA and to the RRE, these antibiotics have been also reported to interfere with splicing (104) and to bind to hammerhead ribozymes (104). The binding affinities of the aminoglycosides for their RNA targets are not very high, they are in the low μM range (40, 101, 104, 105), and therefore not surprisingly this binding has been shown to have a low degree of specificity or selectivity (106). Because of this, it is expected that a large number of cellular RNA molecules will bind to these compounds in the μM range. In fact, aminoglycoside antibiotics are known to be quite toxic to human (107). As with intercalating agents, these molecules are also not very interesting from a therapeutic point of view, due to toxicity and lack of specificity.

4.3.2 Screening approaches

A classical approach towards drug discovery has been the random screening of a vast number of synthetic organic compounds or fungal/plants extracts. This method of discovery, combined with the use of medicinal chemistry, has been very successful in discovering new activities resulting in the development of therapeutic agents. Not surprisingly, this approach has been utilized to discover compounds capable of inhibiting Rev function.

A small-molecule inhibitor of Rev was discovered at Sterling-Winthrop (now Sanofi-Winthrop), using a 96-well plate assay to measure Rev function in transfected cells (108). The assay measured production of the p24 protein from the HIV *gag-pol* gene as a result of Rev expression, in COS-1 cells. A series of structurally related compounds, 8-alkyl-2-(4-pyridyl)pyrido[2,3-*d*]pyrimidin-5(8H)-ones, were found to inhibit Rev-dependent p24

production with an IC_{50} in the low μM range. These compounds were also found to inhibit HIV-1 replication in a human T lymphoma line in the same concentration range. Because cytotoxicity was observed at concentrations of ~25 μM, these compounds are not likely to be of therapeutic use in their present form, although they could be considered leads for the design of less toxic, more potent derivatives. At the same time, this effort has provided proof that inhibitors of Rev can be found using classical screening approaches.

A screening of natural products using an *in vitro* Rev-RRE binding assay was carried out at Bristol-Myers Squibb. Three novel natural products, one from a plant and two from fungi, were discovered and isolated by bio-assay guided fractionation (109, 110). The plant metabolite, niruriside, was isolated from *Phyllanthus niruri*, a plant widely used in Indian traditional medicine. This compound was shown to inhibit binding of Rev to the RRE at an IC_{50} of 3 μM, while the IC_{50} on an unrelated protein-RNA binding system (the R17 coat protein/operator RNA) was greater than 130 μM (109). However , this compound did not protect CEM-SS cells from acute HIV infection (109). Likewise, the two fungal metabolites, harziphilone and fleephilone, from the fungus *Trichoderma harzianum*, were found to inhibit Rev-RRE binding by 50 % at 2-8 μM, but had no anti-HIV activity as tested in the cytoprotection assay (110). Because of this lack of antiviral activity, these natural products are not considered useful. It is not clear why these compounds failed to inhibit HIV replication: since they were discovered in an in vitro assay, it is therefore possible that these metabolites fail to enter the cell, are metabolized by it, or have masking cytotoxic effects. These concerns were not addressed by these publications.

In contrast with Rev-RRE binding approaches, or cell-based assays measuring Rev function, a recent effort to discover inhibitors of Rev has focused on nuclear export (111). Rev acts in conjunction with the cellular nuclear export machinery, and to function it needs to translocate from the nucleus to the cytoplasm (see Sections 3.3 and 3.4). Four antibiotics of the leptomycin-kazusamycin family were found to inhibit the export of Rev to the cytoplasm at nanomolar concentrations, in Rev-expressing HeLa cells treated with actinomycin D. Leptomycin B was found to be specific in its inhibition of the nuclear export pathway, while it had no effect on nuclear import processes (111). This antibiotic was also found to inhibit HIV-1 replication in primary human monocytes, with an IC_{50} of 0.6 nM (111). However, because of its long-term toxicity in tissue culture, leptomycin cannot be used therapeutically. Although this drug was shown to affect only Rev-dependent gene expression, it is possible that the transport of other cellular molecules (proteins or ribosomal or small nuclear RNAs) is also inhibited. The inhibition of a cellular pathway used by Rev could explain the toxic effects of this drug, and it raises the possibility that all Rev inhibitors that affect this Rev function will prove unsuitable as therapeutic agents. A greater knowledge of the nuclear export pathway used by different cellular protein and mRNA species will be necessary to evaluate this hypothesis.

Although none of the compounds discovered to inhibit Rev function is currently being pursued as potential drugs, it

is important to point out that drug discovery is a laborious and sometimes slow process, and that Rev has only recently become a target for discovery and development. At Oncogene Science, Inc., we are carrying out high throughput screening seeking Rev inhibitors, using a cell-based assay similar to the one used at Sterling-Winthrop (108). This program is in the early phases, and we hope to contribute new entities with new activities.